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MINI-REVIEW

NADH Oxidase of Plasma Membranes

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Abstract

NADH oxidase is a cyanide-resistant and hormone-responsive oxidase intrinsic to the plasma membrane of both plant and animal cells. The activity has many unique characteristics that distinguish it from other oxidases and oxidoreductases of both organelles and internal membranes and from other oxidoreductases of the plasma membrane. Among these are resistance to inhibition by cyanide, catalase, superoxide dismutase, and phenylchloromercuribenzoate. Activity is stimulated by hormones and growth factors and inhibited by quinone analogs such as piericidin, the flavin antagonist atebrin, and growth inhibiting gangliosides such as G_{M3}. In marked contact to the NADH-ferricyanide oxidoreductase of the plasma membrane, the NADH oxidase is activated by lysophospholipids and fatty acids, products of phospholipase A_2 action, in a time-dependent manner suggestive of stabilization of an activated form of the enzyme. The hormone-responsive NADH oxidase of the plasma membrane is not a peroxidase and may function as a terminal oxidase to link transfer of electrons from NADH to oxygen at the plasma membrane. The functional significance of the NADH oxidase of the plasma membrane is unknown but some relationship to growth or growth control is indicated. In both animal and plant plasma membranes, the oxidase is activated by growth factors and hormones to which the cells or tissues of origin have functional hormone or growth factor receptors. In addition, substances that inhibit the oxidase, the associated transmembrane reductase or both, inhibit growth. In transformed cells and tissues, the hormone and growth factor responsiveness of the NADH oxidase is reduced or absent. With human keratinocytes which exhibit an increased sensitivity to the antiproliferative action of both retinoic acid and calcitriol, the NADH oxidase of the plasma membrane is strongly inhibited by these agents and shows the same increased sensitivity. If transfer of electrons from NADH to oxygen across or within the eukaryotic plasma membrane is an important aspect of growth or growth control, then the hormone- and growth factor-responsive NADH oxidase associated with the plasma membrane could be of fundamental importance. Because of its low basal activity, stimulation by growth

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factors and hormones, and the inhibition of growth in direct proportion to inhibition of the oxidase, the activity is a candidate as a rate-limiting step in the growth process. Completely unknown is the mechanism whereby NADH oxidization and growth or growth control may be coupled. This, together with further characterization of the activity and the mechanism of loss of control with neoplastic transformation, represent important challenges for future investigations.

Key words: NADH oxidase; plasma membrane; signal transduction; plasma membrane redox enzymes; oxygen; growth transformation; ubiquinone; membranes; growth factors.

Introduction

Transmembrane transport of electrons from cytoplasmic NADH to impermeant artificial electron acceptors, such as ferricyanide, is a ubiquitous property of eukaryote plasma membranes (Crane, 1985; Crane and Barr, 1989). However, if transplasma membrane electron transport is to serve a physiological function, then a natural electron acceptor for NADH oxidation at the plasma membrane, most likely oxygen, would be required.

An enzymatic transfer of electrons from reduced pyridine nucleotide (NADH) to molecular oxygen in the absence of added electron acceptors which defines NADH oxidase activity has been identified through analyses of isolates of plasma membrane highly purified by aqueous two-phase partition from both animals and plants. The activity appears to exhibit some relationship to growth control and to fulfil a function as a terminal oxidase of plasma membrane electron transport (Morré *et al.*, 1988). The oxidase may be coupled to other electron chain constituents via quinones. In this chapter, the characteristics of eukaryotic NADH oxidase will be summarized together with recent evidence for a functional role in the control of growth and/or growth-related processes.

NADH Oxidase Characteristics

The NADH oxidase of mammalian plasma membranes has been characterized extensively from a single source, rat liver. The liver enzyme contains two peptides of M_r ca. 32 and 68 kDa. It is an intrinsic membrane protein and requires detergent (e.g., 2:1 CHAPS:protein, w/w) for solubilization. However, with plant plasma membrane, even with sealed right-side-out plasma membrane vesicles, the activity exhibits a low degree of structure-linked latency indicative of a site for NADH oxidation at the external membrane surface.

NADH oxidase activity may be assayed either as the increase in oxygen consumption induced by addition of NADH as measured with an oxygen

Table I. Loss of NADH Oxidase Activity of Rat Liver Plasma Membrane as a Result of Lipid Extraction and Restoration of Activity with Added Quinone and the Effects on Stimulation of NADH Oxidase by Diferric Transferrin (Fe_2TF) from Crane *et al.*, 1991

Treatment	No Fe_2TF	+ 10 μM Fe_2TF
	nmol/min/mg protein[b]	
Untreated	7.5 ± 0.5	9.8 ± 0.7
Lipid extracted[a]	1.7 ± 0.8	1.8 ± 0.8
+ Ubiquinone	5.5 ± 0.7	8.0 ± 1.2

[a] Lyophylized membranes were extracted with heptane for 7 h at room temperature. To restore activity, coenzyme Q (10 μM) was added in heptane followed by evaporation of the heptane according to the procedure of Norling *et al.* (1974).

[b] Assay conditions were 50 mM Tris-HCl, pH 7.4m 100 μM NADH, 1 mM KCN, and 0.075 mg rat liver plasma membrane in a final volume of 2.8 ml. Activity was estimated from the decrease in absorbance at 340 nm with reference at 430 nm at 37°C using an SLM 2000 spectrophotometer in the dual-beam mode of operation.

electrode, or from the oxidation of NADH as measured spectrophotometrically as the decrease in absorbance of NADH at 340 nm. The stoichometry of NADH oxidized to oxygen consumed by the oxidase of plasma membranes is approximately 2. The pH optimum is near 7. NADH oxidase activity is greatly decreased by extraction of lipid cofactors from the membranes using petroleum ether (Table I). This activity is largely restored by adding back ubiquinone in approximately the same amounts as present in the membrane. Other than NADH and quinones, there are no obvious cofactors for the enzyme. Low levels of Ca^{2+} may be beneficial. Frozen membranes sometimes exhibit a greater calcium response than do fresh membranes.

NADH Oxidase Is Distinct from NADH Oxidase Activities of Internal Membranes

Goldenberg *et al.* (1979) prepared plasma membrane fractions from mouse liver that were > 95% plasma membrane sheets identified morphologically by the presence of junctional complexes. These membranes contained an NADH oxidase activity, with oxygen as acceptor, approximately 25% of that from fractions of endoplasmic reticulum or outer mitochondrial membranes, yet the combined contamination of the plasma membrane by these membranes based on the analysis of marker enzymes was < 5%.

A plasma membrane location of the activity was evidenced by the classic digitonin shift experiment according to Amar-Costesec *et al.* (1974). Because of a high cholesterol content, plasma membranes increase in density when treated with digitonin whereas cholesterol-poor internal membranes do not.

The NADH oxidase activity of plasma membrane preparations was, in fact, shifted from a density of 1.14 to a density of 1.19 when treated with 1 mg digitonin/mg of protein (Goldenberg *et al.*, 1979). In contrast, monoamine oxidase of outer mitochondrial membranes and endoplasmic reticulum markers, e.g., glucose-6-phosphatase, were unaffected by the digitonin treatment.

The oxidase activity of the plasma membrane is insensitive to cyanide to distinguish the NADH oxidase activity of the plasma membrane from that of mitochondrial inner membranes, which is blocked by cyanide. Cyanide (1 mM) is added routinely to assays of plasma membrane NADH oxidase to eliminate possible contributions to the activity from small amounts of

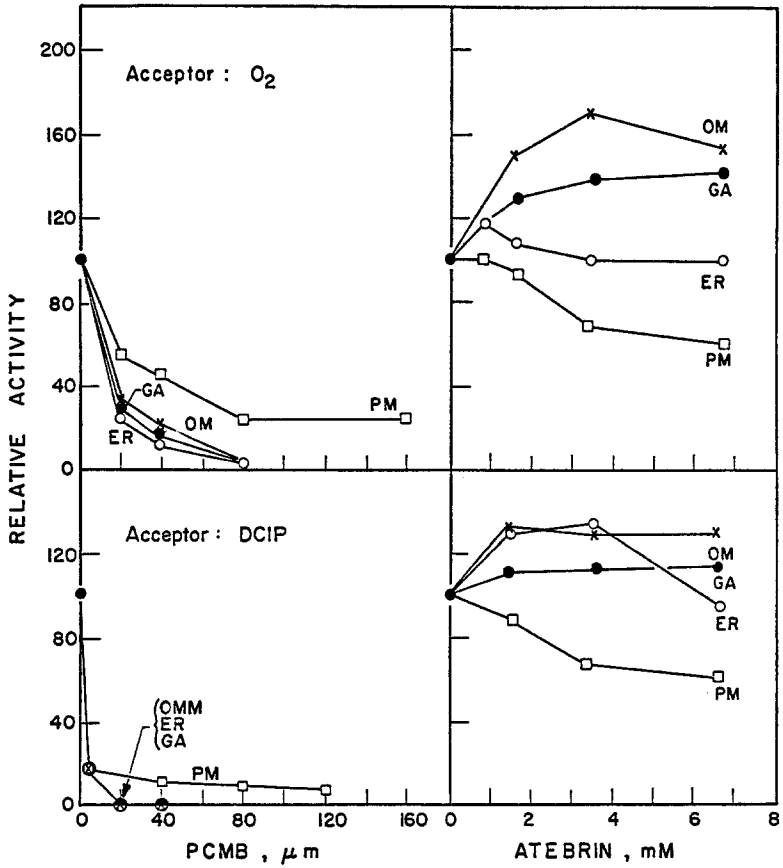

Fig. 1. Effect of atebrin and PCMB on NADH-DCIP reductase and NADH oxidase of mouse liver membranes. The compounds were added to the assays 2 min prior to starting the reaction with NADH. Plasma membrane (PM); endoplasmic reticulum (ER); Golgi apparatus (GA); outer mitochondrial membrane (OM). From Goldenberg *et al.* (1979).

contaminating mitochondria. Rotenone, another inhibitor of the mitochondrial oxidase, also is without effect on the plasma membrane oxidase.

While not absolute as with the mitochondrial inner membrane activity, the NADH oxidase activity can be distinguished from that of endoplasmic reticulum, Golgi apparatus, and outer mitochondrial membranes from its relative insensitivity to the sulfhydryl reagent PCMB and a greater sensitivity to the flavin antagonist atebrin. The activity of endoplasmic reticulum, Golgi apparatus, and outer mitochondrial membranes is inhibited completely at 80 μM PCMB, whereas approximately one-third of the activity of the plasma membrane oxidase remains even at 160 μM PCMB (Fig. 1). Similarly, the NADH oxidase activities of endoplasmic reticulum, Golgi apparatus, and outer mitochondrial membranes are unaffected or stimulated by 2 to 8 mM atebrin, whereas that of the plasma membrane is inhibited (Fig. 1). These data confirm the identification of a NADH oxidase as an enzyme protein unique to the plasma membrane.

The Plasma Membrane NADH Oxidase Is Not a Peroxidase

Hydrogen peroxide can be a product of NADPH oxidation by plasma membranes of rat adipocytes (Mukherjee and Lynn, 1977; Mukherjee *et al.*, 1978). However, only limited amounts of hydrogen peroxide are produced during NADH oxidation by plasma membranes of rat liver (Ramasarma *et al.*, 1981). An NADH oxidase capable of hydrogen peroxide generation has been induced by decavanadate, but not orthovanadate, in a variety of plasma membranes, including those of erythrocytes (Vijaya *et al.*, 1984; Darr and Fridovich, 1984) that lack a conventional NADH oxidase. The findings suggest that the induced enzyme, a peroxidase, is different from the endogenous oxidase.

Similarly, highly purified preparations of plasma membranes of plants contain two oxidase forms designated NADH oxidase I and NADH oxidase II. The type I oxidase normally is assayed at pH 7.0–7.4 with micromolar concentrations of NADH and is cyanide insensitive. The K_m for NADH is 200 μM. The final product of the type I NADH oxidase activity appears to be water based on the stoichometry of 2 NADH molecules oxidized to 1 oxygen molecule reduced. The activity of NADH oxidase I is not inhibited by catalase or superoxide dismutase (Pupillo *et al.*, 1986; Brightman *et al.*, 1988).

On the other hand, when isolated preparations of plant plasma membranes are assayed at pH 5.5–6.0 with 1 mM or more NADH, a second type of activity is observed with characteristics of a peroxidase. The apparent K_m for NADH is about 4 mM (Asard *et al.*, 1987; Vianello and Macri, 1989). The

activity is stimulated by phenolics such as SHAM, coniferyl alcohol, or ferrulic acid and manganese and ferrous ions and is inhibited by cyanide and by catalase (Møller and Bérczi, 1985, 1986; Asard *et al.*, 1987; Vianello and Macri, 1989). Moreover, this activity is inhibited by scavengers of superoxide free radicals such as superoxide dismutase (Asard *et al.*, 1987; Vianello and Macri, 1989) and ascorbate (Vianello and Macri, 1989), suggesting an involvement of $O_2^{\cdot -}$ radicals as well. Thus, there are many lines of evidence to help distinguish what has been designated as NADH oxidase I, which seems to lack peroxidatic activity, from NADH oxidase II with peroxidatic activity. Both NADH oxidase I and NADH oxidase II are membrane associated to further distinguish them from the bulk of the plant peroxidases which are soluble or cell wall-bound.

NADH Oxidase Activity Is Distinct from That of the Transmembrane Reductase

NADH oxidase is an activity distinct from that of the transmembrane oxidoreductase (dehydrogenase) also present in isolated plasma membrane preparations. The transmembrane dehydrogenase, as measured by the ability to reduce added ferricyanide (NADH : ferricyanide oxidoreductase), and the bulk of the NADH oxidase are separated readily by DE-52 ion-exchange chromatography of CHAPS-solubilized plasma membrane proteins (Fig. 2). Additionally, the reductase when further purified by size exclusion chromatography and resolved on SDS-PAGE gave a single major polypeptide band from rat liver plasma membrane of M_r 30–32 kDa. A 43-kDa transplasma membrane reductase was reported previously by Wang and Alaupovic (1978) for erythrocyte which lack NADH oxidase activity. The latter enzyme is unable to oxidize molecular oxygen. The purified oxidase, on the other hand, from rat liver plasma membrane contains two polypeptide bands of M_r ca. 32 and 68 kDa. The purified oxidase does not reduce ferricyanide. With plants, an NADH-acceptor oxidoreductase was purified from plasma membranes as a 27-kDa protein activated by flavin (Luster and Buckhout, 1989). The purified enzyme reduced ferricyanide, duroquinone, juglone, or cytochrome *c* with either NADH or NADPH as reductant. Again, molecular oxygen was not reduced by this enzyme to distinguish it from the NADH oxidase which shows a spectrum of molecular weights similar to that for the rat liver plasma membrane (Brightman *et al.*, 1988).

The reductase and oxidase of plants and animals show many differential responses to drugs and inhibitors. Generally, the reductase is more strongly inhibited by adriamycin, cis platinum, bleomycin, and other antitumor agents (Morré and Crane, 1990) than is the oxidase, whereas the oxidase is

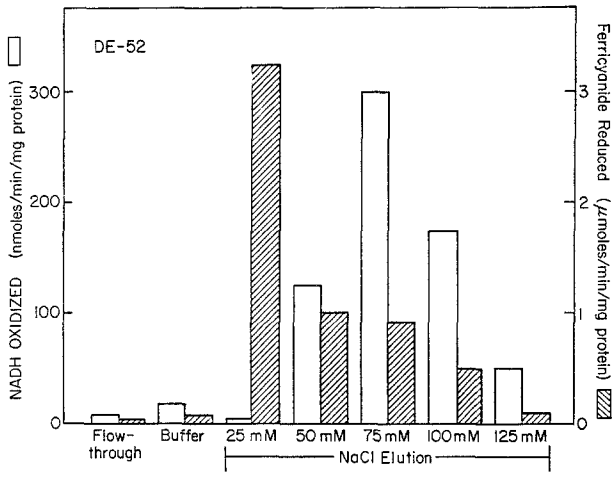

Fig. 2. DE-52 ion-exchange chromatography of purified plasma membranes from rat liver illustrating separation of the transmembrane dehydrogenase (hatched bars) and the NADH oxidase (open bars). CHAPS (0.03%)-solubilized membranes were applied to a DE-52 ion-exchange column and activities were eluted with varying concentrations of sodium chloride. The highest specific activity of ferricyanide oxidoreductase was found in the fractions eluted by 25 mM sodium chloride whereas the NADH oxidase activity eluted as a broad peak centered at 75 mM sodium chloride.

especially sensitive to inhibition by quinone analogs (Table II). The reductase is strongly inhibited in HL-60 cells by retinoic acid (Morré *et al.*, 1991b), whereas the oxidase is not. In contrast, the oxidase is strongly inhibited by extremely low concentrations of calcitriol (Morré *et al.*, 1991b). However, the most definitive characteristics that distinguish the reductase and oxidase are

Table II. Effects of Coenzyme Q Analogs on NADH Oxidase Activity of Rat Liver Plasma Membrane and the Stimulation of NADH Oxidase by Diferric Transferrin (Fe_2TF)[a] (Unpublished Data of I. L. Sun, Purdue University)

	No Fe_2TF	+ 17 μM Fe_2TF
Addition	nmol/min/mg protein	
Control	2.0	6.5
Piericidin A (10^{-7} M)	0.3	0.3
HDMEQ 30 (μg/ml)	0.0	0.0
DMNMQ 24 (μg/ml)	28.5	36.9
Coenzyme Q_{10} (10 μM)	5.7	13.2
Piericidin A (10^{-7} M) + Coenzyme Q_{10} (10 μM)	1.5	2.7

[a]HDMEQ = 6-hexadecyl-2 methoxy-3-ethoxy-5 methyl-1,4-benzoquinone; DMNMQ = 6-naphthylmercapto-2,3 dimethoxy-1,4 benzoquinone. Assay as described for Table I.

hormone and growth factor responsiveness and activation by lysophospholipids and free fatty acids.

NADH Oxidase Stimulated by Growth Factors and Hormones

While the NADH oxidase of plasma membrane preparations has been known for some time as NADH oxidation measured in the absence of added external acceptors (e.g., Gayda *et al.*, 1977), the identification of NADH oxidase as a unique enzymatic activity of plasma membranes came as a result of studies with hormone-responsive plasma membrane preparations from plants. Studies with NADH oxidation in the presence or absence of ascorbate radical revealed an accelerated NADH oxidation in response to a synthetic growth hormone (Morré *et al.*, 1986). The activity stimulated, however, was electron transfer from NADH to oxygen, i.e., NADH oxidase and not ascorbate radical oxidoreductase. NADPH oxidase also is not hormone stimulated and is presumed to represent a different activity.

With the plant membranes, the auxin growth factors, low-molecular-weight substances that promote both cell expansion and initiate cell division in plants (Morré and Key, 1967), stimulated the oxidase directly 2.0- to 2.5-fold, precisely the same degree to which plant cell elongation was stimulated by auxin (Fig. 3). Both the naturally occurring auxin (indole-3-acetic acid or IAA) and synthetic auxin analogs (2,4-dichlorophenoxyacetic acid = 2,4-D or α-naphthaleneacetic acid = α-NAA) stimulated the activity. Inactive hormone analogs (2,3-D or β-NAA) or benzoic acid were without effect on the oxidase (Fig. 3).

To further characterize the hormone stimulation, the oxidase activity was purified from detergent-solubilized plasma membranes (Brightman and Morré, 1988). The detergent solubilized and partially purified activity (Fig. 4) retained the capacity to be hormone stimulated (Brightman and Morré, 1988).

The initial step for any hormone-initiated event is the binding of hormone to receptor. An auxin receptor, or auxin-binding protein (ABP), has been purified from shoot tissues of maize seedlings by several laboratories (Löbler and Klämbt, 1985a, b; Venis, 1986; Shimomura *et al.*, 1986; Löbler *et al.*, 1987). The relative molecular weights of the ABPs are in the range of 20–22 kDa and are localized in the endoplasmic reticulum. Incubation of shoot tissues with antibodies to maize ABP inhibited elongation in the presence of auxin, suggesting the surface exposure of ABPs since IgG molecules cannot cross the plasma membrane (Löbler and Klämbt, 1985b). These same antibodies inhibit the NADH oxidase of plasma membranes from seedling soybean shoots (results unpublished) and seedling maize shoots to approximately

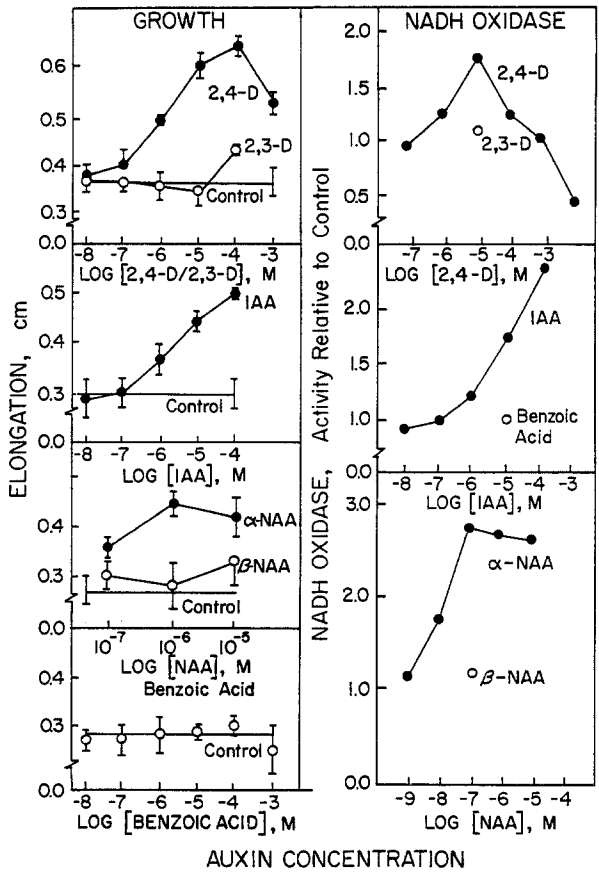

Fig. 3. Stimulation of elongation growth and NADH oxidase activity by auxins. Growth was measured as elongation of 1-cm segments of soybean hypocotyls. NADH oxidase activity was of isolated plasma membrane of soybean. Active auxins, indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (α-NAA), and their inactive analogs, 2,3-D, β-NAA, and benzoic acid, were compared. Values are averaged of three different experiments (10 segments/datum point/experiment) ± standard deviations among experiments for growth of 1-cm hypocotyl segments and from determinations with at least two different plasma membrane preparations for NADH oxidase. Note that the stimulation of NADH oxidase was specific for growth-stimulatory auxins and the concentration curves for stimulation of growth and NADH oxidase were similar.

the same extent as growth (Löbler and Klämbt, 1985b). These findings offer additional evidence for the close linkage of the auxin receptor and the NADH oxidase in plants. With plant plasma membranes, the NADH oxidase activity is the only plasma membrane redox activity that is hormone responsible (Table III). Electron transfers from NADH to external impermeant acceptors (ferricyanide, ascorbate, cytochrome *c*) are not hormone sensitive.

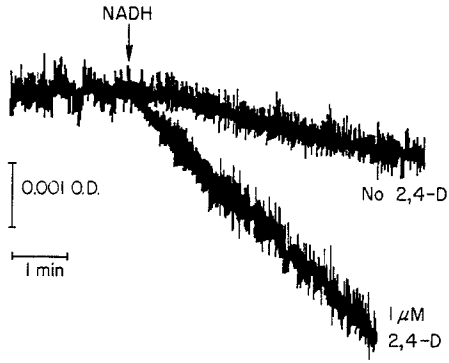

Fig. 4. Spectrophotometric tracing of purified NADH oxidase activity and stimulation by 2,4-D. The sample was incubated with or without 1 μM 2,4-D for 3 min prior to addition of NADH. Control activity (no 2,4-D) was 9.4 nmol/min/mg protein. From Brightman *et al.*, 1988.

Table III. Effect of Auxin on NADH-Dependent Redox Activities of Soybean Plasma Membranes[a]

Electron Acceptor	E^0, pH 7 (V)	Specific activity		Auxin effect (%)
		Control	+ Auxin	
Oxaloacetate	(−0.166)	656	566	(−14)
Ascorbate radical	(+0.058)	3.1	3.3	(+9)
Cytochrome *c*	(+0.240)	7.9	7.8	(−1)
Ferricyanide	(+0.360)	309	273	(−12)
Nitrate	(+0.421)	0.4	0.4	(0)
Oxygen	(+0.815)	0.7	1.6	(+123)

[a]All activities were determined as the oxidation of NADH (decrease in absorbance at 340 nm) in the presence of electron acceptor. Auxin (1 μM 2,4-D) was added to the reaction after an initial rate had been established. Specific activity units are nmol/min/mg protein. Unpublished data of A. O. Brightman.

Table IV. Growth Factor Stimulation of NADH Oxidase Activities of Plasma Membranes from Rat Liver (Unpublished Results of D. J. Morré and A. O. Brightman, Purdue University)

Additions	nmol/min/mg protein
None	0.72
+ 27 nM EGF	1.08
+ 5 μg/ml Insulin	1.20
+ 2.5 μl/ml Pituitary extract	1.48
+ 10^{-7} M Retinoic acid	0.54
+ 10^{-10} M Calcitriol	0.16

With liver plasma membranes, the NADH oxidase exhibits a similar responsiveness to hormones and growth factors and is stimulated approximately 2-fold by epidermal growth factor, transferrin, insulin, and vasopressin (Table IV), see also Tables I and II). The NADH oxidase of adipocyte plasma membrane is stimulated by insulin (Mukherjee and Lynn, 1977; D. M. Morré *et al.*, 1989) as well.

NADH Oxidase Response to Growth Factors Altered by Transformation

The growth factor-responsiveness of the NADH oxidase of plasma membranes prepared from transformed cells appears much reduced from that of their nontransformed counterparts. During hepatocarcinogenesis, the specific activity of the NADH oxidase of plasma membranes increases but growth factor sensitivity declines. With plasma membranes isolated from hyperplastic liver nodules, the NADH oxidase is no longer responsive to transferrin (Table V). This is not due to a reduction in the density of transferrin receptor which actually is increased on plasma membranes of hyperplastic liver nodules. Plasma membranes isolated from transplanted

Table V. NADH Oxidase of Plasma Membrane from Rat Liver and Hyperplastic Nodules of the Rat (from Morré *et al.*, 1991a)

Tissue	nmol/min/mg protein
Liver	0.8 ± 0.1
+ 3 μM Diferric transferrin	1.3 ± 0.02
Hyperplastic nodules	1.5 ± 0.1
+ 3 μM Diferric transferrin	1.6 ± 0.1

Table VI. Response of the NADH Oxidase of Plasma Membranes from Human Keratinocytes (Normal or Human Papilloma Virus (HPV)-Transformed) to Various Growth Factors (Unpublished Data of D. J. Morré, D. M. Morré, Kim Creek, and L. A. Pirisi)

Addition (100 sec)	After 100 sec Followed by (100 sec)	Normal	HPV-transformed
		nmol/min/mg protein[a]	
None		9.2 ± 1.6	4.5 ± 2.5
+ 27 nM EGF		17.9 ± 1.4	4.6 ± 0.1
+ 27 nM EGF	+ 10^{-7} M Retinoic acid	9.7 ± 0.6	2.3
+ 27 nM EGF	+ 10^{-10} M Calcitriol	9.8 ± 0.6	2.4 ± 0.1
+ 10^{-10} M Calcitriol		7.4 ± 1.4	3.3 ± 0.3
+ 10^{-10} M Calcitriol	+ 10^{-7} M Retinoic acid	0.6 ± 0.3	0.6 ± 0.2

[a]Corresponding rates for NADH-ferricyanide oxidoreductase activities comparing plasma membranes from normal and HPV-transformed cells were 580 ± 15 and 366 ± 48 nmol/min/mg protein, respectively.

hepatomas (RLT-28) exhibit NADH oxidase activities about twice that of liver but the oxidase is less responsive to transferrin, insulin, and epidermal growth factor than is the oxidase of liver plasma membrane.

With human keratinocytes grown in culture, the NADH oxidase of isolated plasma membranes is stimulated by epidermal growth factor (Table VI). However, with immortalized keratinocytes transformed by human papilloma virus, the oxidase fails to respond. These findings point to the interesting possibility that the coupling of the NADH oxidase to hormone and growth factor receptors is reduced or lost in parallel to the loss of growth control that is characteristic of neoplastic transformation.

Growth Inhibitory Gangliosides Inhibit NADH Oxidase

Simple gangliosides, such as the monosialoganglioside G_{M3}, are inhibitory to the growth of cultured mammalian cells (Keenan *et al.*, 1975). Gangliosides are concentrated in the outer leaflet of the plasma membrane (Op den Kamp, 1979; Weigandt, 1985). As such, their growth effects also should be most pronounced on membrane proteins of the outer membrane leaflet. An inhibitory effect of gangliosides on platelet-derived (PDGF) (Bremer *et al.*, 1984) and epidermal (EGF) (Bremer *et al.*, 1986) growth factor receptor responses has been indicated for growth factor-stimulated phosphorylation or autophosphorylation. NADH oxidase also is inhibited by growth inhibitory gangliosides in a ganglioside-specific (Table VI) and dose-dependent (Morré and Crane, 1990) manner. These inhibitory effects of gangliosides on NADH oxidase are given both with isolated plasma membranes and with the partially purified NADH oxidase of rat liver. The inhibition of the NADH oxidase by gangliosides argues for an external location of the oxidase, although other explanations such as mediation of inhibition via an interaction with an external glycolipid receptor or transducin-like molecule also are possible.

NADH Oxidase Activity and Growth Control

A number of arguments have been put forth in support of a relationship between plasma membrane redox activities and control of growth. These include inhibition of redox activities by growth-inhibitory drugs (Sun and Crane, 1984, 1985; Sun *et al.*, 1987a), and the actual stimulation of cell growth by artificial electron acceptors (Ellem and Kay, 1983; Sun *et al.*, 1985a, b). Artificial electron acceptors have been shown to directly stimulate the growth of melanoma cells (Ellem and Kay, 1983) and HeLa cells (Sun

et al., 1985a, b) and to partly replace the essential components of calf serum. However, artificial electron acceptors such as ferricyanide, ferric complexes, hexamine ruthenium II, or indigotetrasulfonate (Crane *et al.*, 1985) are not normal constituents of the external cellular milieu, whereas oxygen is. In the absence of external electron acceptors, NADH oxidase may serve as a rate-limiting terminal oxidase to facilitate transfer of electrons to oxygen.

Recent evidence implicating NADH oxidase in the control of animal cell growth has come from studies of antiproliferative effects of retinoic acid and calcitriol. Proliferation of normal neonatal foreskin keratinocytes grown in culture is inhibited at about 10^{-6} M retinoic acid, and the cells of the

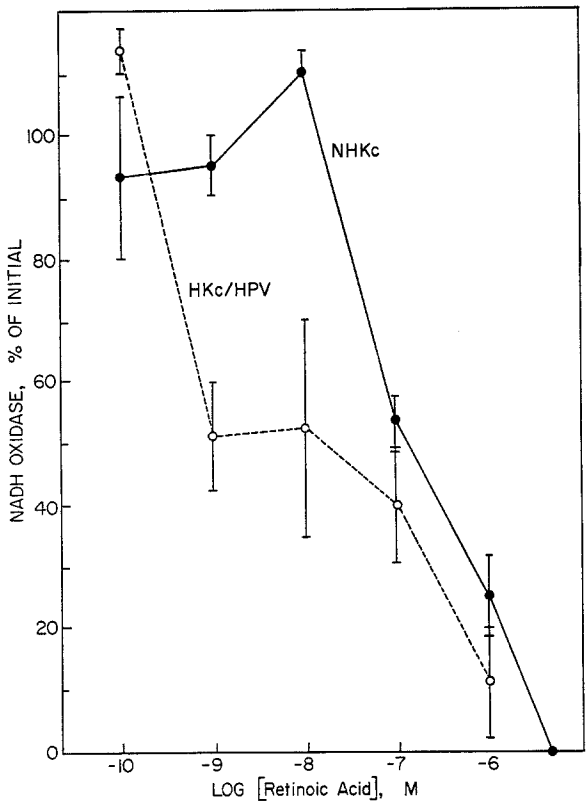

Fig. 5. Inhibition of NADH oxidase of human neonatal foreskin keratinocytes in culture by retinoic acid. The NADH oxidase of human keratinocytes transformed with human papilloma virus (HKc/HPV) are inhibited at much lower concentrations of retinoic acid (dotted curve, open symbols) than is the NADH oxidase of their normal counterparts (NHKc; solid line and symbols). With the transformed keratinocytes, growth is inhibited by retinoic acid at much lower concentrations as well. Unpublished data of D. J. Morré, D. M. Morré, K. E. Creek, and L. A. Pirisi.

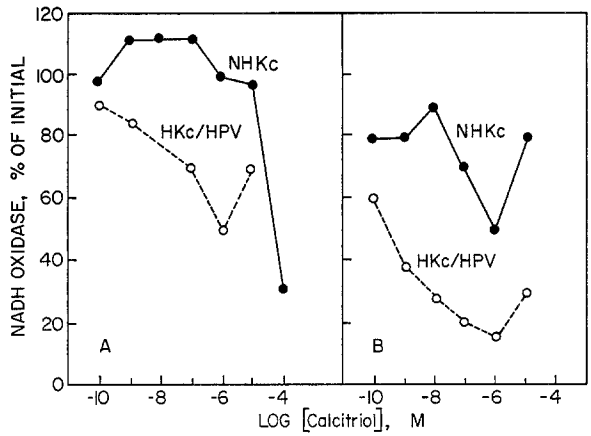

Fig. 6. Inhibition of NADH oxidase of human neonatal foreskin keratinocytes in culture by calcitriol (1,25-dihydroxy vitamin D_3). The results shown in A and B compare two different plasma membrane preparations. In both, the NADH oxidase of the keratinocytes transformed with human papilloma virus (HKc/HPV) exhibits a much greater sensitivity to inhibition by the calcitriol than do the normal (NHKc) keratinocytes. Unpublished data of D. J. Morré, D. M. Morré, K. E. Creek, and L. A. Pirisi.

growth-inhibited cultures subsequently differentiate to form envelopes. However, with keratinocytes transformed with human papilloma virus (HPV), growth is inhibited at about 10-fold lower concentrations of retinoic acid. With isolated plasma membranes, not only is NADH oxidase inhibited by growth-inhibitory concentrations of retinoic acid but the plasma membranes from the virus-transformed cells are inhibited at 10-fold lower concentrations of retinoic acid than are their normal counterparts (Fig. 5).

Similarly, with calcitriol, a substance with nuclear receptors of the same gene family as retinoic acid (Petkovich *et al.*, 1987), cell proliferation is blocked (McLane and Katz, 1988; McLane *et al.*, 1990). In normal keratinocytes, NADH oxidase is inhibited by calcitriol at similar concentrations as is growth (Fig. 6). However, with human papilloma virus-transformed keratinocytes, the NADH oxidase is 50% inhibited by calcitriol concentrations as low as 10^{-10} M (Fig. 6B). This increased sensitivity to antiproliferative substances, together with reduced responsiveness to hormones and growth factors, add interest to the NADH oxidase as a potential drug target for development of new antitumor agents.

Plant growth has been reported to be inhibited by reduced O_2 levels under conditions where ATP production in mitochondria would not be expected to be growth-limiting (Atwell *et al.*, 1984; Griffin, 1968). This might be interpreted as additional evidence for a role of NADH oxidase in the control of plant cell elongation. Oxygen is a limiting factor for growth in

mammalian cells (Miller *et al.*, 1987) but any relationship between oxygen requirements for growth and plasma membrane NADH oxidase have not been investigated.

NADH has been identified as the natural electron donor for plasma membrane electron transport in HeLa cells promoted by artificial electron acceptors (Navas *et al.*, 1986). NADH levels together with oxygen supply rate have been implicated as important to cell proliferation (Schwartz *et al.*, 1974).

NADH Oxidase as Part of an Electron Transport Chain

If the oxidase is to function as a terminal oxidase to facilitate transfer of electrons to O_2 as the natural electron acceptor, then the oxidase and the oxidation of cytoplasmic NADH must somehow be coupled. A number of possibilities may be considered by which this coupling of NADH oxidation and transfer of electrons to oxygen might occur.

One possibility is that the oxidase is either a transmembrane protein or is located on the cytoplasmic membrane surface and, therefore, accessible to NADH. Using plasma membrane vesicles having a predominantly cytoplasmic side-in orientation, the oxidase is inhibited by the gangliosiee G_{M3}, is inhibited by impermeant drugs such as actinomycin D, and shows low structure-linked latency when the vesicles are disrupted by detergent. These observations do not support a cytoplasmic orientation for the plasma membrane oxidase but, rather are properties suggestive of an external or transmembrane orientation. When right-side-out and inside-out vesicles prepared by free-flow electrophoresis are compared, both show NADH oxidase of comparable specific activities. Therefore, the oxidase appears to have the ability to accept electrons from NADH from either surface of the plasma membrane despite the fact that physiological access to NADH must be via the cytoplasm.

Alternatively the oxidase might be linked to cytoplasmic NADH via other redox constituents to form a chain. The NADH-ferricyanide oxidoreductase is somehow linked to the cell surface in order to reduce external ferricyanide and other impermeant ferric complexes such as ferric ammonium citrate or ferric ions carried by diferric transferrin (Sun *et al.*, 1987b). The NADH-ferricyanide oxidoreductase, however, of itself appears unable to transfer electrons to oxygen, so one alternative could be to couple the two activities. Even from theoretical considerations, it is expected that a minimum of three prosthetic groups would be necessary to bridge 35 Å of membrane lipid bilayer because of 10 Å limitations on electron tunneling (Williams and Conear, 1986).

Flavin, iron, copper, and thiols all have been identified in some plasma membranes (Cunningham *et al.*, 1982). Cytochromes of the *b* type may be

present as well, including both P-420 and P-450 (Jarasch *et al.*, 1979). There also are reports (Jarasch *et al.*, 1979; Crawford and Schneider, 1982; Lutter *et al.*, 1984), recently confirmed (Crane *et al.*, 1991), concerning the presence of coenzyme Q. Other possible candidates as intermediate acceptors would be pterins (da Costa and Rothernberg, 1988), non-heme iron, ascorbate free radical (Sun *et al.*, 1984; Morré *et al.*, 1985, 1987) and various organic free radical-generating groups. An involvement of plasma membrane P-450 in plasma membrane electron transport from NADH is regarded as unlikely due to a lack of carbon monoxide inhibition of the bulk of the transmembrane activity and the preference of this enzyme for NADPH.

The oxidase activity is inhibited by *N*-ethylmaleimide and is responsive to sulfhydryl protectants (A. Hidalgo, A. Brightman, P. Navas, and D. J. Morré, unpublished results). These findings suggest some role for sulfhydryl groups in the activity of the oxidase and/or in the linkage between the reductase and an electron transport chain.

As mentioned above, several lines of evidence indicate a role for quinones. NADH oxidase activity of plasma membranes is reduced when quinones are extracted from the membrane and the activity is restored by the addition of coenzyme Q (Table I). Even the basal activity is stimulated by addition of coenzyme Q to the membranes. Additionally, NADH oxidase is markedly inhibited by various quinone analogs such as piericidin A (Table II).

Activation by Products of Phospholipase A Activity

The lipid environment of the membrane has effects, both direct and indirect, on the control of enzymatic activities potentially important to growth. One such example is provided by the 1,2-diacylglycerols from phosphoinositides generated through the action of phospholipase C which activate C-type protein kinases (Berridge, 1978). The response of protein kinases (Parkos *et al.*, 1987) and the NADH oxidase to positive control by lysophospholipids and fatty acids (Fig. 7), products of the action of A-type phospholipases on phospholipids, are yet other examples. With the NADH oxidase, stimulations of several hundred percent are readily achieved by both lysophospholipids and fatty acids (Fig. 8, Table VII). The NADH oxidases of both rat liver and of a plant, soybean, are stimulated. The stimulations by lysophospholipids and free fatty acids occur under latency-free conditions of 0.1%. Triton X-100 (Table VIII) to rule out a simple detergent effect. In contrast, the NADH-ferricyanide oxidoreductase activity of either liver or soybean plasma membrane exhibits a different pattern of stimulation in response to products of phospholipase A action. There is no apparent activation beyond an initial stimulation attributed to structure-linked latency

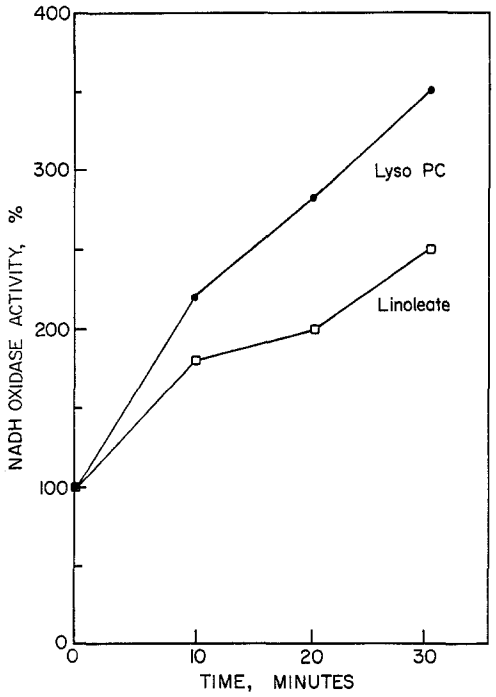

Fig. 7. Stimulation of NADH oxidase of rat liver plasma membranes by lysophosphatidylcholine (Lyso PC) (20 μM) and by a free fatty acid (linoleic acid) (200 μM) and the time dependence of stimulation. The stimulation of NADH oxidase by both the lysophospholipid and the fatty acid increases with time of incubation. The unstimulated rate was 2.35 $\pm$ 0.10 nmol/min/mg protein. Such a response would be consistent with an activation mechanism based upon stabilization of an active conformation of the enzyme. Similar results were obtained in the presence of 0.1% Triton X-100 (e.g., Table VIII) to eliminate structure-linked latency as the basis for the stimulatory response. Unpublished data of A. Brightman, X. Z. Zhu, and J. Wang, Purdue University.

Table VII. Response of Redox Activities of Plasma Membranes Purified from Rat Liver to Gangliosides Prepared from Bovine Brain[a]

	Percent of no ganglioside	
Ganglioside (10^{-5} M)	NADH oxidase	NADH-Ferricyanide oxidoreductase
Monosialoganglioside, G_{M3}	50	92
Disialoganglioside, G_{D1a}	85	82
Trisialoganglioside, G_{T1B}	105	92
Mixed brain gangliosides	70	88

[a]Note the preferential inhibition of the NADH oxidase by the monosialoganglioside G_{M3}. Unpublished data of D. J. Morré, F. E. Wilkinson, L. Y. Wu, and X. Z. Zhu, Purdue University.

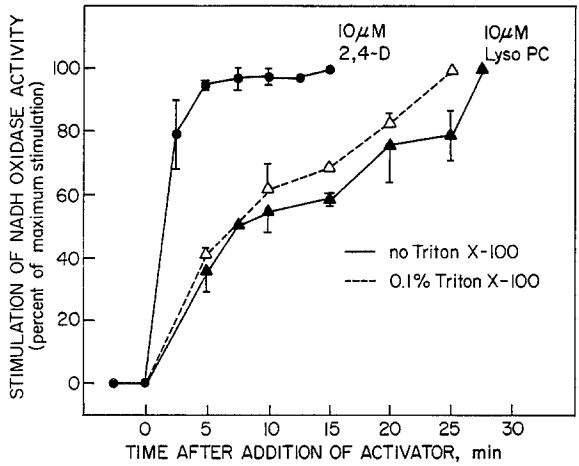

Fig. 8. Kinetics of stimulation of NADH oxidase of plasma membranes of dark grown soybean stems by auxin and lysolipid. Maximum stimulation of NADH oxidase by auxin was reached within 5 min of addition of the growth regulator to the enzyme reaction. In contrast, lysophosphatidylcholine activated the enzyme more slowly than auxin, reaching a maximum in approximately 30 min of incubation. Incubation in the presence of 0.1% Triton X-100 to solubilize NADH oxidase did not increase significantly the activation rate by lysophosphatidylcholine. The control activity was 0.80 ± 0.13 nmol/min/mg protein.

resulting from the location of the active site on the inner or cytoplasmic surface of plasma membrane vesicles. The time-dependent activation of NADH oxidase by lysolipids and free fatty acids under latency-free conditions is another characteristic to distinguish the plasma membrane oxidase from the plasma membrane reductase.

The enhancement of NADH oxidase by lysophospholipids and fatty acids in a time-dependent manner (Fig. 8) suggest a mechanism whereby enzyme may be stabilized by the lipid products in a more reactive conformation (Scharff *et al.*, 1983). The time-dependent enhancement by lipids differs from hormone activation which is direct and occurs without a perceptible time lag.

Table VIII. Stimulation of NADH Oxidase Activity of Rat Liver Plasma Membrane by 30 μM Lysophosphatidylcholine or Linoleic Acid in the Presence or Absence of 0.1% Triton X-100 (Unpublished Data of A. Brightman and X.-Z. Zhu, Purdue University)

Additions	0.1% Triton X-100	nmol/min/mg protein
None	−	1.5
	+	1.6
Lysophosphatidylcholine	−	5.3
	+	5.8
Linoleic acid (30 μM)	−	3.4
	+	5.3

Thus, the hormone-dependent activation of NADH oxidase *in situ* may involve more than one type of control. In the first instance, the oxidase appears to be coupled in some manner to functional hormone or growth factor receptors at the cells' surface. If the plasma membrane has functional (coupled) receptors for a particular growth factor or hormone, it would appear that the NADH oxidase will be stimulated or inhibited in a manner that parallels the growth response to the particular growth factor or hormone. Additionally, the hormone-dependent activation of a phospholipase A_2, for example, linked to a GTP-binding protein (Jelsema, 1987), may result in a further stimulation of the oxidase due to the time-dependent stimulation by the lipase products.

At present, the significance of possible interactions between the NADH oxidase and products of phospholipase A action has been little studied. However, in plants, both lysophospholipids and free fatty acids (when supplied as their methyl esters) stimulate hormone-induced growth with approximately the same concentration dependence as they stimulate NADH oxidase activity. While it is clear that such correlations might very well be circumstantial, they add to the evidence that the NADH oxidase activity of the plasma membrane and growth control are linked.

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